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Research Article

Application and Validation of Simple Isocratic HPLC-UV-DAD Method with Dual Wavelength Detection for Ivabradine Determination and Its Application in the Study of Stress Degradation

Joanna Nowakowska,[1] Piotr Pikul,[1,2] Marcin Marszałł,[3] and Krzesimir Ciura[1]

[1]*Department of Physical Chemistry, Medical University of Gdańsk, Gdańsk, Poland*
[2]*Department of Therapy Monitoring and Pharmacogenetics, Medical University of Gdańsk, Gdańsk, Poland*
[3]*Department of Toxicology, Medical University of Gdańsk, Gdańsk, Poland*

Correspondence should be addressed to Krzesimir Ciura; krzesimir.ciura@gmail.com

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Ivabradine is a modern drug that selectively lowers the heart rate, improves cardiac energy balance, and reduces heart's demands for oxygen and energy. Due to the chemical nature of ivabradine, which absorbs light at 207 nm and 286 nm, its detection was performed at two wavelengths. A Knauer C8 column was used to develop the RP-HPLC method for determination of ivabradine. The proposed method was linear from 5 to 100 μg/ml ($r > 0.999$) for both wavelengths and limits of detection (LOD) and limits of quantification (LOQ) were 0.33 and 1.09 μg/ml for 207 nm and 1.19 and 3.97 μg/ml for 286 nm, respectively. After validation, the investigated method was applied to a stress degradation study. Numerous degradation products were formed from ivabradine solutions through alkaline and acid hydrolysis, oxidation, and photolysis. The largest numbers of degradation products were found in the sample exposed to 24 h radiation and alkaline hydrolysis (eight and six products, resp.). Finally, the simple method using HPLC-UV-DAD was developed and validated. Its usefulness for the monitoring of possible degradation products was demonstrated.

1. Introduction

Ivabradine is a cardiac drug that belongs to the group of funny channel (I_f) inhibitors [1]. It is the only representative of that group of drugs that has been introduced for the treatment of chronic coronary artery disease and chronic heart failure [2, 3]. This molecule selectively blocks the f channel, which is responsible for initiation of the diastolic depolarisation phase of the action potential, resulting in slower increase of the pacemaker current. The slower the increase of pacemaker current, the lower the heart rate. Because of this mechanism, ivabradine has been effectively introduced into cardiac therapeutics. I_f channels, also called hyperpolarisation-activated cyclic nucleotide-gated (HCN) channels, are intermembrane structures that act as nonselective ligand-gated cation channels. There are four known HCN channels, labelled from 1 to 4. HCN4 is the most widespread isoform in the sinoatrial node and is responsible for diastolic depolarisation [4–6].

The drug is orally administrated in doses of 5 or 7.5 mg as its hydrochloride salt. It should also be emphasised that ivabradine (brand name Procoralan) is produced by only one company, Servier. For this reason, ivabradine does not have a pharmacopoeia monograph. As a consequence, the reference method for ivabradine determination is not universally accepted. Ivabradine often appears in the scientific literature, but a monograph describing the method of its determination is still not sufficient. Studies about the application of thin layer chromatography (TLC) to its analysis have been published in the last five years [7, 8]. The most important HPLC work was carried out by Klippert et al. [9, 10]. They applied fluorescence and mass spectroscopy as detectors but neither method of detection is so widespread as UV-DAD detection.

Stress testing of the drug substance may help in identification of probable degradation products, which can lead to a better understanding of the degradation pathways and the internal stability of the molecule. Our previous work presented forced degradation studies of ivabradine [11]. The analyses were carried out with HPLC-MS. Other researchers have also presented investigations of ivabradine degradation products. Mass spectroscopy detection has been widely used in identification of ivabradine [12, 13]. Patel et al. have studied ivabradine degradation products with Q-TOF LC/MS. During the conducted tests, only five degradation products were identified from acid degradation [14]. Degradation products of ivabradine have also been examined with the use of TLC [15]. HPLC with UV-Vis detection has been used to establish degradation products of ivabradine, but the authors obtained from its acid hydrolysis only one degradation product [16]. The main purpose of this work is to develop and validate a simple method for the quantitative determination of ivabradine, based on HPLC-UV-DAD, and to show its applicability for detection of degradation products and possible impurities.

2. Experimental

2.1. Instrumentation. The UV-Vis diode array detector UVD340S, pump P 580, column thermostat STH 585, autosampler injector ASI-100, and operating system Chromeleon, all from Dionex Corporation (Sunnyvale, CA, USA), were used to perform chromatographic analysis operated by the Chromeleon chromatography management system. The Knauer C8 column (5 μm, 250 mm × 4.6 mm ID) was used during this study.

Degradation studies were carried out using Thermostat CC2-K6 from Huber (Offenburg, Germany); photo stability studies were performed utilizing a photostability Suntest CPS+ Atlas chamber (Accelerated Tabletop Exposure Systems, Gelnhausen, Germany) with a xenon lamp.

2.2. Chemicals and Reagents. The tested ivabradine hydrochloride (purity ≥ 99%) bulk powder was supplied by Watson International Ltd. (Kunshan, China). Acetonitrile was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA); ammonium acetate, hydrochloric acid, and sodium hydroxide (analytical grade) were purchased from POCH (Gliwice, Poland). 30% hydrogen peroxide was bought from J. T. Baker (Deventer, The Netherlands). Before use, water was purified by Direct-Q3 UV-R Merck Millipore system (Darmstadt, Germany).

2.3. Chromatographic Conditions. The mobile phase consisted of 40% acetonitrile and 60% 20 mmol ammonium acetate aqueous solution. The isocratic flow was set at 1 ml/min and the injection volume was 20 μl. The column oven was set at 40°C and the autosampler temperature at 4°C. Detection of ivabradine was performed at 207 and 286 nm. Average retention time was 8.42 (±0.12) min.

2.4. Method Validation. Validation was performed for two wavelengths: 207 and 286 nm. The 207 nm wavelength is rarely used, due to the unstable baseline, but these studies have shown its usefulness. The specificity of the method was proved by determining peak purity for IVA in a mixture of stressed samples using HPLC-UV-DAD. To establish linearity, solution containing 1 mg/ml of IVA was diluted to six different concentrations within the range of 5–200 μg/ml. All samples were analysed in triplicate. The accuracy was determined by analysis of samples at three different concentrations (10, 50, and 100 μg/ml) in triplicate, and the recoveries of the added drug were obtained from the difference between peak areas of fortified and unfortified samples. The intra- and interday precisions were determined at three different concentrations, 10, 50, and 100 μg/ml, on the same day (n = 3) and on 3 consecutive days (n = 9). The robustness of the proposed method was determined by introducing the purposeful changes in the flow rate (0.6–0.8 ml/min), column temperature (30 ± 5°C), and pH of mobile phase (3.0 ± 0.2), while analysing samples at three different concentrations (10, 50, and 100 μg/ml). Each sample was injected in triplicate (n = 3) and obtained peak areas were used to calculate mean and % RSD values.

Stability studies of the stock solution showed no changes in ivabradine concentrations after 24 h and 48 h storage at 4°C, −20°C, and −80°C. The mobile phase was also stable under stock solution storage conditions, as well as the analysis conditions.

Limit of detection was determined based on the standard deviation of response (mean SD value of y-intercept) and the slope of the calibration curve. The limit of quantification was considered to be three times the limit of detection.

The detection limit was calculated using the method of the signal to noise ratio. Noise was measured at ±0.5 min from peak start and end for the lowest point from calibration curve. The ratio of signal to noise was determined, and the LOQ was considered to be $3 \times S/N$ while the LOD was $10 \times S/N$.

2.5. Stress Studies. The stress studies were carried out in the same way as described in our previous report [11]. In short, in order to prepare each sample, 1 mg of ivabradine was weighed and dissolved in 2 ml of the appropriate solvent. Thermal degradation was performed by addition of deionised water and storing for 24 h at 80°C. For acid and alkaline hydrolysis, 2 mol/l HCl or 1 mol/l NaOH was added to samples and incubated for 24 h at 80°C. Studies of possible oxidation products were carried out by adding 3% H_2O_2, 7.5% H_2O_2, and 15% H_2O_2 and incubating for 24 h at 80°C. Photolytic degradation was performed in solution of deionised water for 24 h and 48 h and in solid form for 120 h. The illuminance was set at 500 W/m^2.

3. Results and Discussion

3.1. Method Development. In order to determine the best conditions for the analysis, optimisation of the method was performed. In the first step, columns of different properties were investigated: Hypersil Gold C8, 5 μm, 150 mm × 4.6 mm ID; Zorbax, 5 μm, 150 mm × 4.6 mm ID; Knauer C8, 5 μm, 250 mm × 4.6 mm ID; and Supelco C18, 5 μm, 250 mm ×

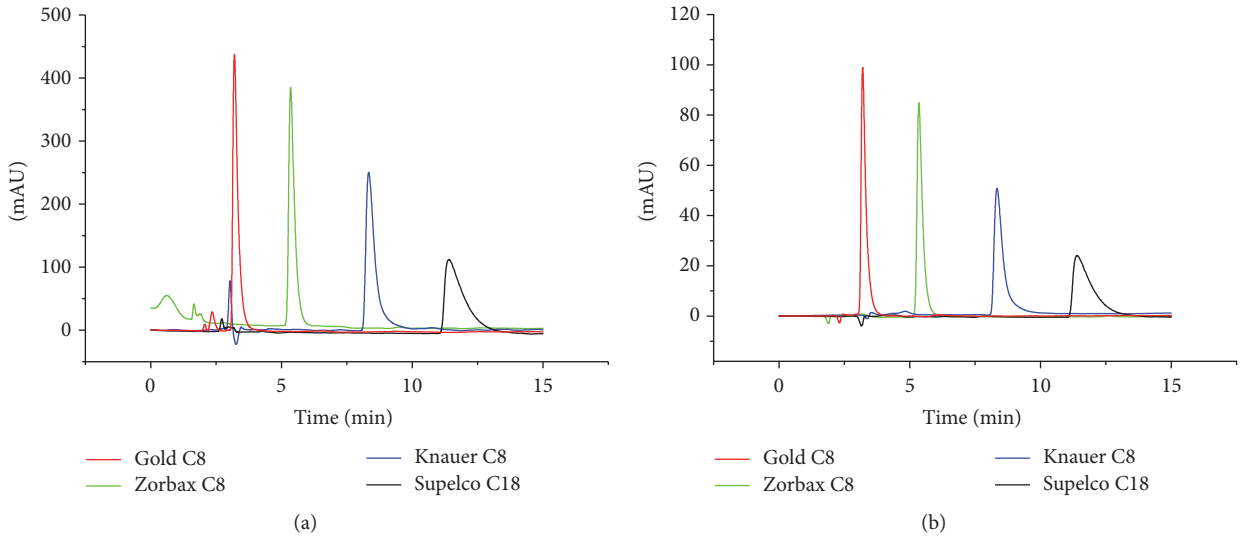

Figure 1: Optimisation of columns for (a) 207 nm and (b) 286 nm.

Table 1: The obtained retention factor, peak asymmetry, and numbers of theoretical plates of investigated columns.

	207 nm			286 nm		
	Retention factor (k)	Peak asymmetry	Theoretical plates (HEPT)	Retention factor (k)	Peak asymmetry	Theoretical plates (HEPT)
Gold C8	0.62	2.18	0.102	0.63	2.15	0.087
Zorbax C8	2.57	1.89	0.050	2.01	1.82	0.044
Knauer C8	2.20	2.20	0.076	1.85	2.31	0.074
Supelco C18	3.43	3.22	0.193	2.86	3.50	0.194

4.6 mm ID To choose the most appropriate column, the retention factor, peak asymmetry factor, and height equivalent to a theoretical plate (HETP), as well as the retention time of ivabradine, were taken into consideration (Table 1). The obtained chromatograms are presented in Figure 1. Hypersil Gold C8 was not suitable for ivabradine determination because of the low retention factor (0.62 and 0.63). Supelco C18 was also not suitable due to its high HETP (0.193 and 0.194). The retention time of IVA should be long enough to allow the determination of stress products, which would probably be of lower molecular weight than ivabradine; therefore they would interact less with the column, resulting in a shorter retention time [16]. Both Zorbax C8 and Knauer C8 columns were appropriate for analysis of ivabradine, but it was decided to perform validation on the Knauer C8 column. In the next step, the temperature of the column during analysis was optimised. The results, summarised in Table 2, showed that the temperature has an impact on peak asymmetry. The lowest peak asymmetry was found for the analysis performed with the column oven set to 40°C. Mobile phase conditions were also optimised; both composition (Figure 2) and flow were examined. The best peak shape was observed for a mobile phase consisting of 40% acetonitrile and 60% 20 mmol ammonium acetate. The highest value of HETP was found for a flow rate of 1 ml/min; for 0.8 ml/min the HETP was two times lower, and for 1.2 ml/min it was smaller by nearly 25%. Peak asymmetry was also the lowest for a flow rate of 1 ml/min, regardless of the detection wavelength used.

Table 2: Peak asymmetry for each temperature and wavelength.

	207 nm	286 nm
	Peak asymmetry	Peak asymmetry
35°C	2.70	2.63
38°C	2.64	2.55
40°C	2.55	2.47

Finally, validation and analysis of the degradation products were performed on the Knauer C8, 5 μm, 250 mm × 4.6 mm ID column, with 40°C oven temperature and a mobile phase composed of 40% acetonitrile and 60% 20 mmol ammonium acetate. The flow rate was 1 ml/min.

3.2. Validation Study. In order to prove the usefulness of the method for the determination of ivabradine, the proposed method was validated. The analytical wavelengths 207 nm

	207 nm		286 nm	
	Peak asymmetry	Theoretical plates (HETP)	Peak asymmetry	Theoretical plates (HETP)
32% ACN	2.67	0.102	2.66	0.098
35% ACN	2.71	0.096	2.73	0.092
40% ACN	2.50	0.101	2.36	0.097

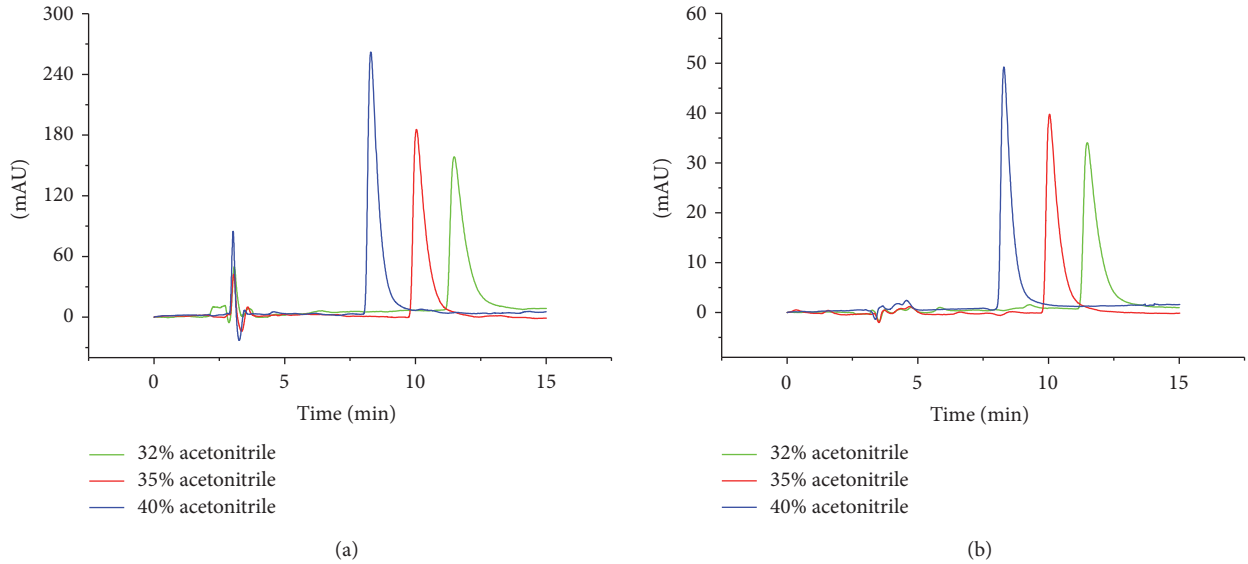

Figure 2: Optimisation of the mobile phase for the Knauer C8 column for 207 nm and 286 nm, with information about peak asymmetry and height equivalent to a theoretical plate, for various amounts of acetonitrile in the mobile phase.

Table 3: Validation parameters of proposed method.

Parameter	207 nm	286 nm
Linear work range [μg/ml]	5–100	5–100
Slope ± SD	1.3286 ± 0.110	0.2544 ± 0.001
Intercept ± SD	−0.3355 ± 0.277	0.0659 ± 0.012
Coefficient of correlation (r)	0.9990	0.9993
Limit of detection (LOD) [μg/ml]	0.33	1.19
Limit of quantification (LOQ) [μg/ml]	1.09	3.97
Intra-assay precision 1 (% RSD, $n = 6$; 10 μg/ml)	5.97	8.23
Intra-assay precision 2 (% RSD, $n = 6$; 50 μg/ml)	5.81	1.60
Intra-assay precision 3 (% RSD, $n = 6$; 100 μg/ml)	6.80	1.01
Interassay precision (% RSD, $n = 3$; 10 μg/ml)	3.44	5.66
Recovery (%, $n = 3$)	92.1–102.2	95.6–109.5

Intra-assay precision concentrations were 10, 50, and 100 μg/ml, respectively.

and 286 nm were selected, since ivabradine has two absorption maxima. It was decided to use both wavelengths for HPLC validation due to the fact that the molar absorption coefficient (ε) for 207 nm is over six times higher than that for 286 nm. As a result, the peak areas at 207 nm were considerably higher, which led to improvement in limit of detection (LOD) and limit of quantification (LOQ) values.

During the validation, it has been recommended to use wavelengths longer than 240 nm [17]. The use of wavelengths less than 240 nm is associated with detection of impurities instead of the analysed compound, but in this case, the use of a short wavelength in the UV range is desirable, since the purpose is conducting the degradation studies. Such an approach increases the probability of detection of degradation products (unknown compounds). As previously shown, ivabradine has two chromophore groups that absorb light in the UV-Vis region, and it is not known which group will be eliminated or changed in the process of degradation, so it was decided to use both mentioned wavelengths [18].

Regression coefficients of the calibration curves, as well as limits of detection and quantification, are shown in Table 3. The method of determination LOQ and LOD based on the

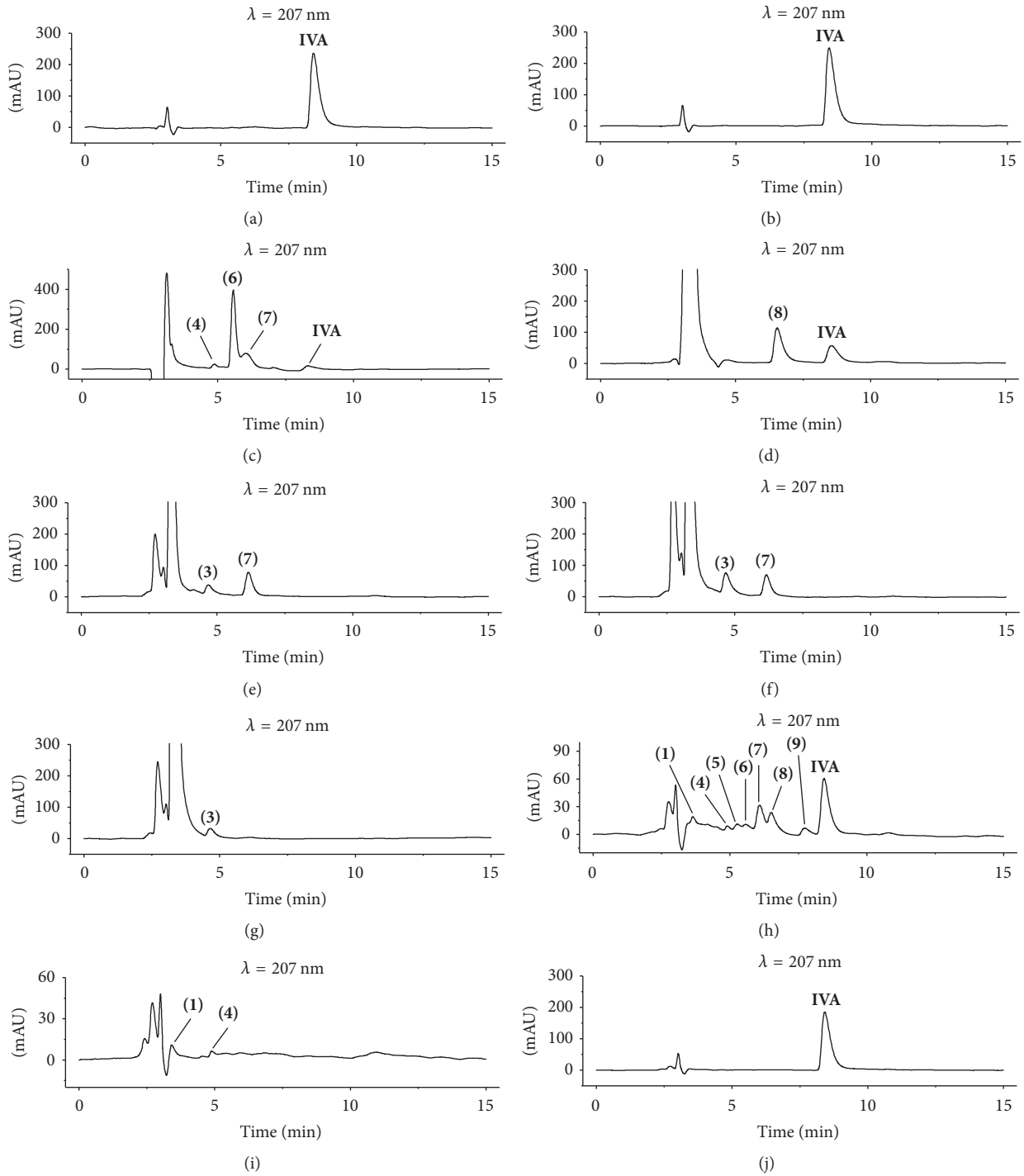

Figure 3: Chromatographic set showing degradation products generated in various conditions at 207 nm: (a) thermal degradation (solution), (b) thermal degradation (powder), (c) acid degradation, (d) alkaline degradation, (e) oxidation (3% H_2O_2), (f) oxidation (7.5% H_2O_2), (g) oxidation (15% H_2O_2), (h) photolysis (24 h), (i) photolysis (48 h), and (j) photolysis (powder, 120 h).

S/N ratio is consistent with the standard deviation of the y-intercept of the calibration curve method, since both give similar results.

Comparing validation parameters of the available methods of ivabradine determination to the UV-Vis detection method, this research had shown wider linearity range than Maheshwari et al., who designated linearity for 286 nm [16]. LOQ and LOD were proved to be very low [16]. However, the wider linearity range for 285 nm was reported in the work by Rahman et al. [19]. A better indirect precision was acquired, probably because of significantly higher concentrations of analyte used [19]. In the research, a wider linearity range

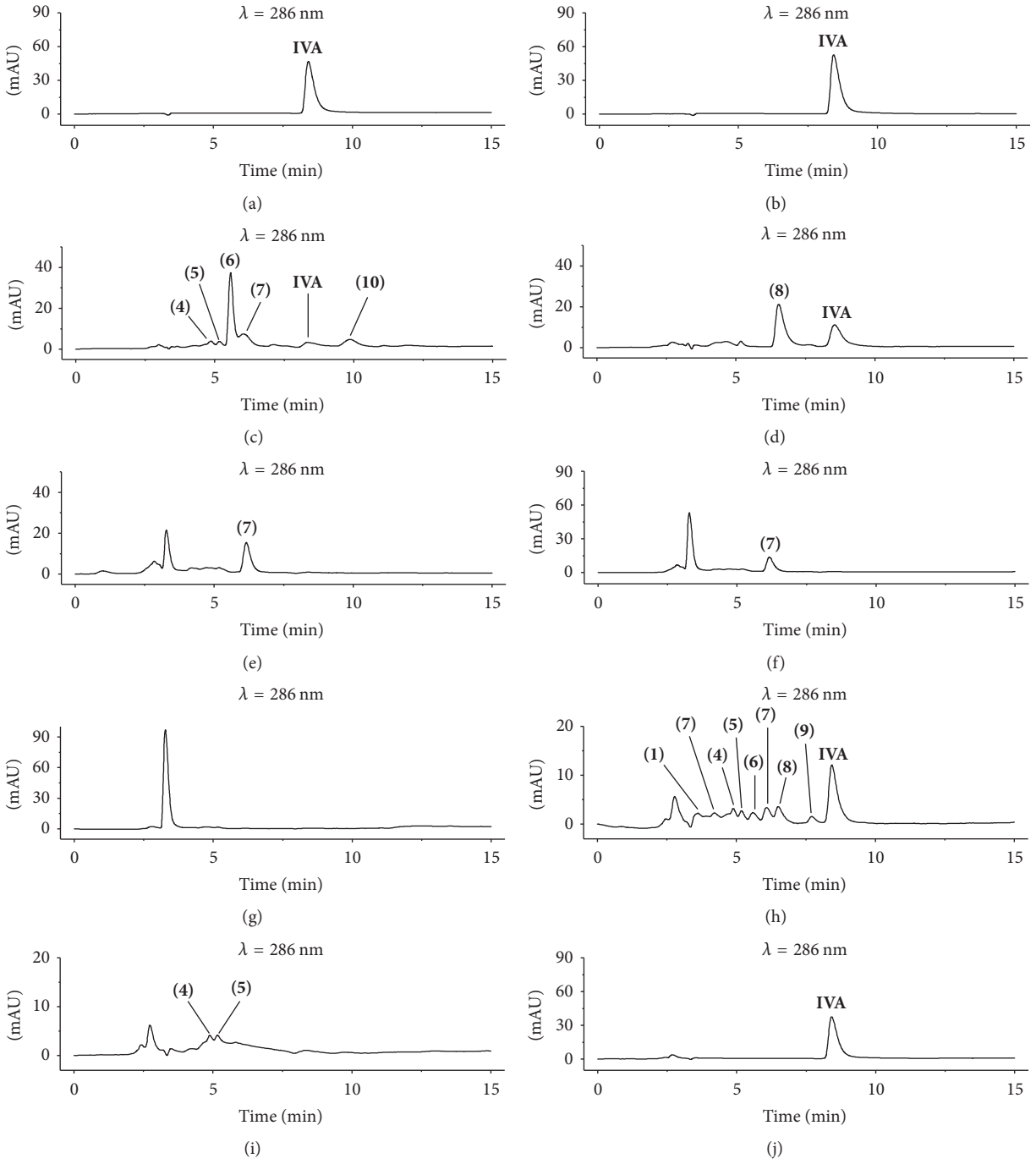

Figure 4: Chromatographic set showing degradation products generated in various conditions at 286 nm: (a) thermal degradation (solution), (b) thermal degradation (powder), (c) acid degradation, (d) alkaline degradation, (e) oxidation (3% H_2O_2), (f) oxidation (7.5% H_2O_2), (g) oxidation (15% H_2O_2), (h) photolysis (24 h), (i) photolysis (48 h), and (j) photolysis (powder, 120 h).

was acquired compared to that in the research carried out by Maheshwari et al. as well as a smaller range than that shown in the paper by Rahman et al. [16, 19]. In both papers, the authors do not mention the method of calculating LOQ and LOD; therefore, it is impossible to compare these values. In both studies retention time did not differ significantly from that obtained in this study.

3.3. Application of Validated Method on Degradation Study. Our previous study discussed widely the stress degradation process of ivabradine. The study was performed with the use of LC-MS/MS apparatus. Generally, LC-MS/MS technology is becoming more popular, but LC-UV/DAD is still the common standard. LC-UV/DAD provides a relatively low cost of maintenance and depreciation of equipment, in comparison

to LC-MS/MS. For this reason, we decided to transfer our method to LC-UV/DAD apparatus. We managed to achieve equally good separation of ivabradine from its degradation products as those described by us in the previous work [11]. Briefly, the largest numbers of degradation products were found in samples exposed to 24 h radiation (eight products) and alkaline hydrolysis (six products). Chromatograms showing ivabradine degradation products are presented in Figures 3 and 4.

The most important aspect is the fact that method could be used for simple separation, as well as detection, of degradation products obtained. From a practical point of view, the UV-DAD detector is widely available for controlling the degradation products of ivabradine in the pharmaceutical industry.

4. Conclusion

A simple isocratic RP-HPLC method for determination of ivabradine and its degradation products was successfully validated. The studies showed that 207 nm meets the conditions required for analytical wavelength. The proposed method is suitable for the determination of ivabradine and its degradation products and could be successfully implemented in quality control and routine analysis of pharmaceutical dosage forms. Various degradation products were obtained, but it can be stated that ivabradine is a stable chemical compound, especially in solid form, from which no degradation products were obtained.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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